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Polyphyly of "Sclerosponges" (Porifera, Demospongiae) Supported by 28S Ribosomal Sequences

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Abstract. To test the competing hypotheses of polyphyly and monophyly of "sclerosponges," sequences from the 5′ end of 28S ribosomal RNA were obtained for *Astrosclera willeyana, Acanthochaetetes wellsi,* and six other demosponge species. Phylogenetic relationships deduced from parsimony and neighbor-joining analyses suggest that these sclerosponges belong to two different orders of Demospongiae: *Astrosclera willeyana,* being closely related to the Agelasidae, belongs to the Agelasida, *Acanthochaetetes wellsi,* being closely related to the Spirastrellidae, belongs to the Hadromerida. These results contradict the hypothesis that sclerosponges are monophyletic and imply that a massive calcareous skeleton has evolved independently in several lineages of sponges.

Introduction

Recent sponges generally have a skeleton made of spicules that are either siliceous (classes Demospongiae and Hexactinellida) or calcareous (class Calcarea). However, 16 living species build an unusual solid calcareous skeleton, which bears a striking similarity to that of various Cnidaria, in addition to this spicular skeleton. These "coralline sponges" are believed to be the survivors of the stromatoporoids, sphinctozoans, and chaetetids, important ancient reef builders that were highly diversified during the Paleozoic and Mesozoic eras and that were long thought to be extinct (Hartman and Goreau, 1970; Vacelet, 1983; Wood, 1990).

Since the discovery of these living coralline sponges, they have been classified according to three systems, each reflecting a different belief in the number of times that sponges have invented a massive calcareous skeleton. In the first, all of the coralline sponges are included in the class Ischyrospongiae (Termier and Termier, 1973). In the second, the massive calcareous skeleton is believed to have evolved at least twice, once among coralline sponges with similarities to the Calcarea and once among coralline sponges that more closely resemble the Demospongiae; the latter group is assigned to the class Sclerospongiae (Hartman and Goreau, 1970). This second interpretation has been the most widely used, appearing in many recent treatises on zoology (Parker, 1982; Riedl, 1983) and paleontology (Rigby and Stearn, 1983). A third system (Vacelet, 1979, 1985) reflects the assertion that the massive calcareous skeleton is more plastic and has evolved in several different lineages within the Demospongiae and Calcarea. Under this system, living and, where possible, fossil coralline sponges are classified within the various taxa of Demospongiae and Calcarea with which they share derived characters.

Three coralline sponges are included in this study: *Acanthochaetetes wellsi* Hartman and Goreau, 1975; *Astrosclera willeyana* Lister, 1900; and *Petrobiona massiliana* Vacelet and Lévi, 1958. *Acanthochaetetes wellsi* and *Astrosclera willeyana* are of special interest, because

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they are considered to be living representatives of chaetetids and stromatoporoids, two groups of great importance in the fossil record. The affinities of these groups were previously uncertain, but they were most often classified in the Cnidaria (Lecompte, 1956; Fischer, 1970). The separation of the two groups implies an independent derivation of the massive calcareous skeleton. The spicular and cytological characters of both species strongly resemble those found in well-defined families of non-calcified demosponges. The choanocytes of *Acanthochaetetes wellsi* possess a periflagellar sleeve; a central cell at the apopyle of the choanocyte chambers, as in the Hadromerida; and a spicule complement similar to that of the family Spirastrellidae in the order Hadromerida (Hartman and Goreau, 1975; Vacelet and Garrone, 1985; Reitner and Engeser, 1987; Boury-Esnault *et al.*, 1990). *Astrosclera willeyana* has small choanocyte chambers, flattened choanocytes, verticillate acanthostyles, and chemical affinities with the order Agelasida (Hartman and Goreau, 1970; Vacelet, 1981; Boury-Esnault *et al.*, 1990; Williams and Faulkner, 1996). *Petrobiona massiliana* has morphological affinities with the class Calcarea.

In this work we generate a new, independent data set based on DNA sequences, use it to construct phylogenies, and compare these with morphological ones. Our main objective is then to determine which of the three hypotheses are consistent with the molecular phylogeny.

Materials and Methods

Material: selection and preservation

The species analysed and their sites of collection are listed in Table 1. Some demosponge species were selected as representatives of the various taxa supposedly related to *Astrosclera* and *Acanthochaetetes.* Other species with various levels of distance from the in-group were chosen: these include representatives of other demosponge subclasses—one Ceractinomorpha species (*Halichondria panicea*) and two Tetractinellida species (*Cinachyrella sp.* and *Discodermia polydiscus*)—and of class Calcarea (*Clathrina cerebrum*), with which *Petrobiona massiliana* has affinities. For further convenience, all demosponge species that are not Tetractinellida are grouped under the collective term "monactinellids." All specimens were either preserved in 70% ethanol or deep-frozen in liquid nitrogen and then kept at −80°C, depending on collecting conditions.

DNA processing

Extraction. The total genomic DNA extraction technique was modified from the Simple Fool's Guide to PCR (Palumbi *et al.*, 1991). Less than 0.5 g of tissue was crushed in a sterile mortar after total dehydration (overnight air-dry at +4°C or speed vac) for the alcohol-preserved samples and in liquid nitrogen for the frozen samples. The powder was gently mixed for a few minutes with 500 μl of lysis buffer (Palumbi *et al.*, 1991). Spicules and cellular remains were then removed by centrifugation for 2 min at 13,000 rpm. Digested tissue was purified successively in phenol, phenol-chloroform-isoamylalcohol, and chloroform-isoamylalcohol extractions. Nucleic acids were precipitated with ammonium acetate-isopropanol, followed by a 70% ethanol wash. Total DNA was resuspended in sterile distilled water and its concentration determined by optic density at 260 nm.

Polymerase chain reaction. Two overlapping fragments of the ribosomal RNA gene were amplified using a universal primer and a sponge-specific primer. Primers used were as follows (specificity, orientation, and position of primers in the aligned sequences of Figure 1 follow each sequence): ITS3 5′-GTCGATGAAGAACGCAGC-3′, universal, forward, external 5′; Ep1b′ 5′-GTGGCC-GGGAGAGGCAGC-3′, part of Demospongiae not Tetractinellida, forward, 257–274; Ep2 5′-CTYYGACGTGCC-TTTCCAGGT-3′, Demospongiae, reverse, 303-323; D2 5′-TCCGTGTTTCAAGACGGG-3′, universal, reverse, external 3′.

The fragment "ITS3-Ep2" contains a part of the 5.8S rRNA gene, the ITS2, the C1 domain and half of the D1 domain of the 28S rRNA gene; the "Ep1b′-D2″ fragment contains the other half of the D1 domain, the C2 and the D2 domains of the 28S rRNA gene.

A 50 μl double-stranded PCR reaction mix contains 0.3 μg template DNA, 2.5 μl DMSO, 0.165 m*M* each dNTP, 30 pmol each probe, 1.5 U Taq DNA polymerase (Bioprobe). This reaction mix was overlaid with mineral oil and placed in a Trio-thermoblock thermocycler (Biometra). Cycling conditions are variable for the annealing temperature (Ta): respectively 60°C and 63°C for ITS3-Ep2 and Ep1b′-D2 primer pair. The first cycle is 4 min at 94°C, 2 min at Ta, and 2 min at 72°C; this is followed by 30 cycles each consisting of 1 min at 94°C, 1 min at Ta, and 1 min at 72°C; the reaction is finished by 4 min at 72°C.

After visualization of 5 μl of the reaction on a 1.5% agarose gel, the remaining 45 μl of PCR product was purified by precipitation with ammonium acetate-isopropanol, followed by a 70% ethanol wash. The pellet was then resuspended in 6 μl of sterile distilled water.

The approximate concentration was evaluated visually by electrophoresis of 1 μl of the purified PCR product in a 1.5% agarose gel, and comparison to 1.5 μl of the DNA molecular weight marker VI (Boehringer Mannheim).

Cloning and sequencing. Each PCR fragment was cloned into PCR-Script SK(+) cloning vector (PCR-

Table I

Sponge species sequenced for analysis of phylogenetic relationships among sclerosponges

Classification	Species	Collection locality
DEMOSPONGIAE		
Tetractinellida		
Tetillidae	*Cinachyrella sp.**	New Caledonia
Theonellidae	*Discodermia polydiscus* Bocage, 1870*	Mediterranean sea, 3PP cave, La Ciotat
"monactinellids"		
Axinellidae	*Axinella damicornis* (Esper, 1794)*	Mediterranean sea, La Ciotat
Agelasidae	*Agelas oroides* (Schmidt, 1864)**	Mediterranean sea, La Ciotat
Astroscleridae	*Astroscleral willeyana* Lister, 1900	New Caledonia 1992
	Astrosclera2 willeyana Lister, 1900	New Caledonia 1994
Clionidae	*Cliona viridis* (Schmidt, 1862)*	Mediterranean sea, La Vesse
Spirastrellidae	*Spirastrella cf. coccinea* (Duchassaing & Michelotti, 1874)	Panama, Atlantic coast San Blas Island
Acanthochaetetidae	*Acanthochaetetes wellsi* Hartman & Goreau, 1975	New Caledonia 1992
Halichondriidae	*Halichondria panicea* (Pallas, 1766)*	South West Channel, Aber Wrac'h
CALCAREA		
Clathrinidae	*Clathrina cerebrum* (Haeckel, 1872)**	Mediterranean sea, La Vesse
Petrobionidae	*Petrobiona massiliana* Vacelet & Lévi, 1958**	Mediterranean sea, Anse des Cuivres

* Sequences from Chombard *et al.* (In press).
** Sequences from Lafay *et al.* (1992).

Script SK(+) cloning kit. Stratagene) and sequenced with the T7 Sequencing kit (Pharmacia Biotech) using [33P]-dATP and adding DMSO in the annealing reaction. The internal probe C2′ is used to obtain the middle of the "Ep1b′-D2" fragments, in addition to the vector probes Ks and T3 (C2′ 5′-GAAAAGAACTTTGRARAG-AGAGT-3′, universal specificity, forward orientation, position 483–505 on the aligned sequences of Figure 1).

Each PCR product was sequenced from a minimum of two clones; when contradictions in the sequences of several clones could not be resolved, the corresponding positions were coded according to the UPIAC code. The two strands were sequenced for the main part of the sequence length, with special attention to the D2 domain where strong secondary structures of the molecule cause compressions in the sequence migration. From the two overlapping PCR products, the final sequence was 1104 bp to 1197 bp in length, depending on the species. This fragment corresponds to the 3′ extremity of the 5.8S rRNA (about 108 bp), the Internal Transcribe Spacer ITS2 (between 167 bp and 224 bp), and the four first domains of the 5′ extremity of the 28S rRNA: C1, D1, C2, and D2 (between 816 and 866 bp).

Sequence management and alignment

The MUST package (Philippe, 1993) was used to manage sequences, including registration (with ENTRYSEQ program), alignment (with ED), construction of distance matrices (with NET or from NJ trees), distance calculations and construction of trees with the neighbor joining algorithm (with NJ), matrix comparison (with COMPMAT), and calculation of bootstrap proportions from neighbor joining trees (with NJBOOT). Wherever likely secondary structures were detected, sequences were aligned according to supposed conservation of helices.

PAUP, version 3.1.1 (Swofford, 1991), was also used for construction of trees and calculation of bootstrap proportions, discussed below. In bootstrap calculations, nonmajority nodes were compared in order to explore the robustness of alternative topologies.

The final alignment presented in Figure 1 was obtained by eye using the editor of MUST (ED). The ITS2 (not presented in Fig. 1) and part of the 5′ extremity of the D2 domain (corresponding to positions 575-640, Fig. 1) are very divergent and cannot be aligned in all our samples, thus these regions were not used in the sequence analysis.

Results

Because previously published sequences of 28S rRNA (Lafay *et al.*, 1992) are shorter than ours, two successive analyses were made. The first grouped all species and corresponds to the length published by Lafay *et al.* (Table I); in the second, *Clathrina, Petrobiona,* and *Agelas* were removed so that we could use our total alignable length.

The first analysis included 12 species and 374 bp of

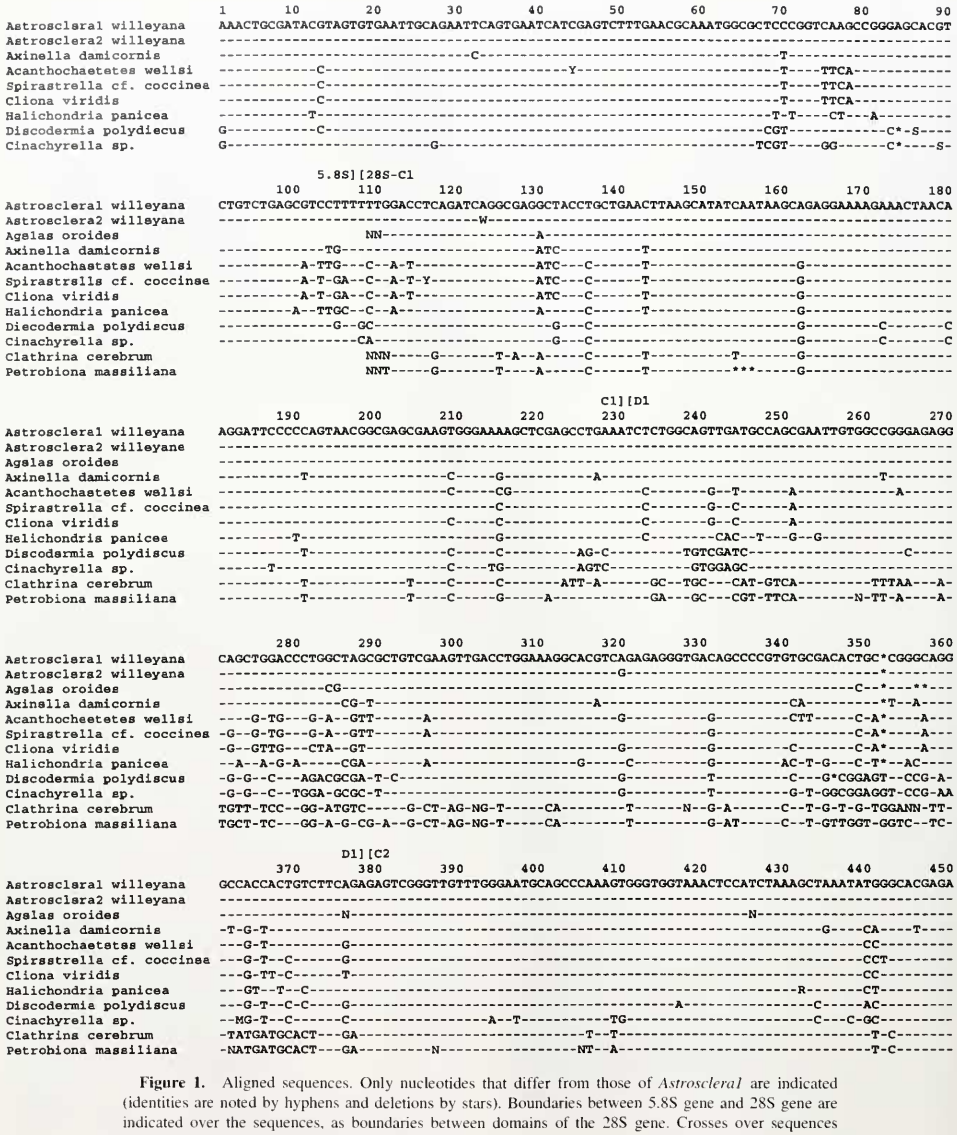

Figure 1. Aligned sequences. Only nucleotides that differ from those of *Astroscleral* are indicated (identities are noted by hyphens and deletions by stars). Boundaries between 5.8S gene and 28S gene are indicated over the sequences, as boundaries between domains of the 28S gene. Crosses over sequences indicate the nonalignable part of the D2 domain, which is not used for phylogenetic analysis.

sequence, 145 of which are variable and 106 informative for parsimony. As shown in Figure 1, these sequences include the C1, D1, and part of the C2 domains of the 28S rRNA gene. Saturation was tested using COMP-MAT of MUST. Global saturation is not detected, observed distances and number of steps inferred by PAUP between

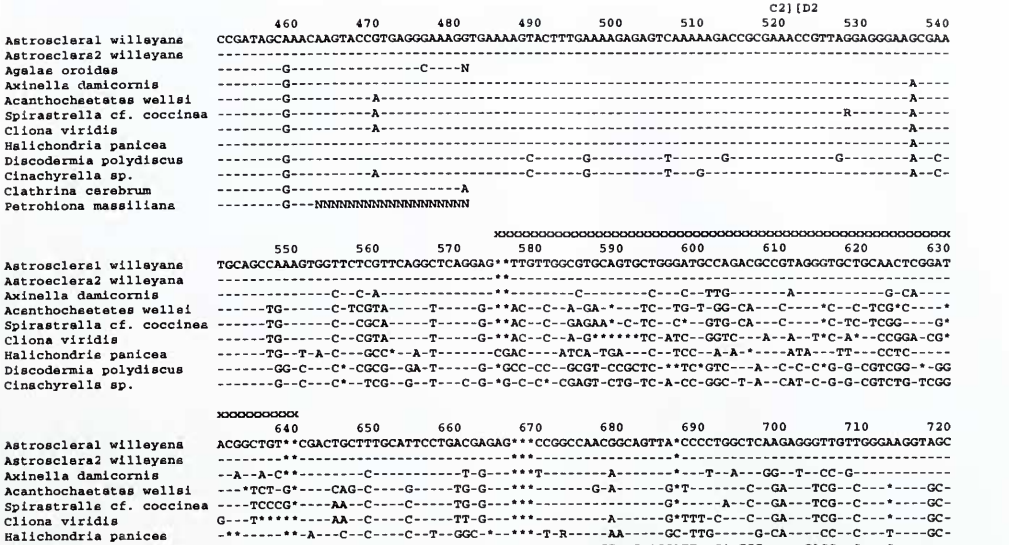

Figure 1. *(Continued)*

all the pairs of species in the data set being linearly correlated (CC 0.98, Fig. 2). Three groups of dots are clearly detectable in this saturation analysis. They correspond to decreasing distances between (1) Calcarea–Demospongiae, (2) Tetractinellida–monactinellids, and (3) monactinellids–monactinellids. The exhaustive search algorithm

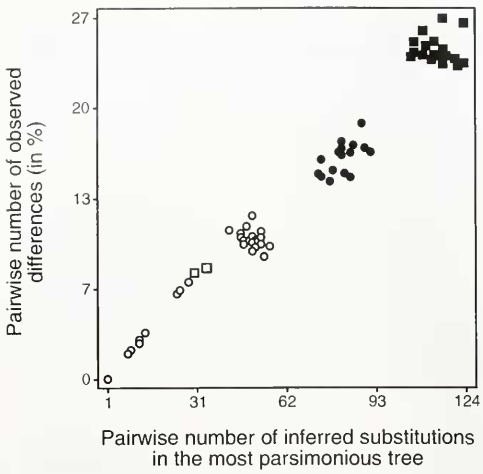

Figure 2. Global saturation curve for 12 species and short-length aligned sequences (374 bp). CC 0.98. White circles are distances between pairs monactinellids-monactinellids. Dark circles are distances between pairs Tetractinellida-monactinellids. Dark squares are distances between pairs Calcarea-Demospongiae. White squares are distances within Tetractinellida and within Calcarea.

of PAUP provided one single shortest tree with 268 steps, a consistency index (CI) of 0.795, a retention index (RI) of 0.767, and a G1 of −1.50. The tree was rooted using the out-group method on both species of Calcarea (*Clathrina cerebrum* and *Petrobiona massiliana*). The resulting single topology is presented in Figure 3. The Branch and Bound search option was used to provide a bootstrap with 1000 replicates in PAUP. The majority-rule consensus

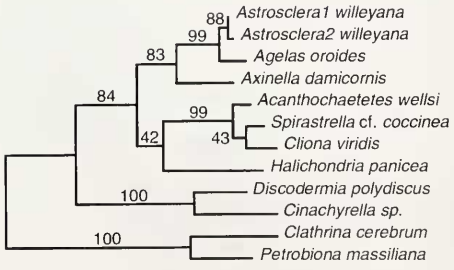

Figure 3. Phylogram obtained with PAUP by exhaustive analysis on short-length aligned sequences (374 bp) for 12 species using ACCTRAN optimization option. Tree length = 268, CI = 0.795, RI = 0.767, and G1 = −1.50. Bootstrap proportions (1000 replicates using Branch and Bound) are shown above internal branches.

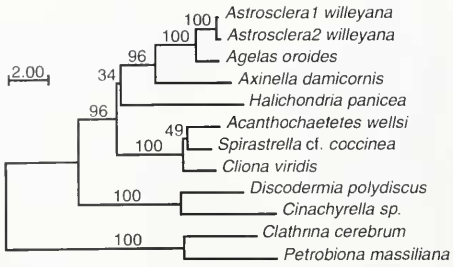

Figure 4. Phylogram obtained with MUST by neighbor-joining analysis on short-length aligned sequences (374 bp) for 12 species. Bootstrap proportions (1000 replicates by NJ analysis) are shown above internal branches.

tree exhibits the same topology as the shortest tree found by exhaustive search (bootstrap proportions [BP] are reported on Fig. 3). The neighbor joining analysis (NJ and NJBOOT in MUST) provided a topology that differs in the location of *Halichondria* and of *Acanthochaetetes*, and in having slightly better bootstrap proportions (Fig. 4).

This first analysis indicates that the Tetractinellida are monophyletic, a conclusion supported by a 100% BP (Chombard *et al.*, in press). This group constitutes the sister group of the other Demospongiae called here "monactinellids." This last group is supported by a 96% BP in distance analysis and an 84% BP in parsimony analysis (Figs. 3–4). All the alternative topologies found by parsimony have less than 5% BP, implying that monactinellids are the monophyletic sister group to the Tetractinellida. For the second analysis of full-length sequences, we are thus able to take the Tetractinellida as an out-group related to the monactinellids. In monactinellids, "sclerosponges" are polyphyletic. *Acanthochaetetes* is included in a hadromerid clade, in which the monophyly of (*Acanthochaetetes, Spirastrella, Cliona*) is supported by respectively 100% BP in distance and 99% BP in parsimony analysis (Figs. 3–4). Relationships within this clade are not strongly supported by this first analysis. *Astrosclera* (two individuals) is included in an axinellid clade, in which the monophyly of (*Astrosclera1, Astrosclera2, Agelas, Axinella*) is supported by 96% BP and 83% BP in distance and parsimony analysis respectively. Unlike the hadromerid clade, the axinellid clade has relationships that are well supported—in particular the monophyly of (*Agelas, Astrosclera1, Astrosclera2*), which is supported by 100% BP and 99% BP in distance and parsimony analysis respectively.

The second analysis was made for 9 species and 914 bp of sequence, 388 of which are variable and 244 informa-

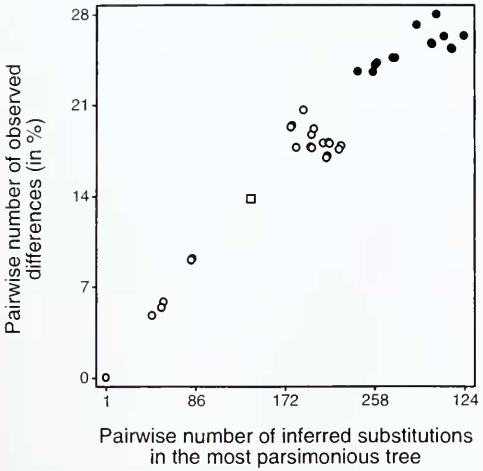

Figure 5. Global saturation curve for 9 species and full-length aligned sequences (914 bp). CC 0.97. White circles are distances between pairs monactinellids-monactinellids. Dark circles are distances between pairs Tetractinellida-monactinellids. White squares are distances within Tetractinellida.

tive for parsimony. No global saturation is evident (CC 0.97, Fig. 5). The exhaustive search algorithm of PAUP provided one single shortest tree with 640 steps, CI = 0.839, RI = 0.783, and G1 = −1.01. The tree was rooted using the out-group method on the tetractinellids (*Cinachyrella sp.* and *Discodermia polydiscus*). The resulting single topology is presented in Figure 6. The Branch and Bound search option was used to provide a bootstrap with 1000 replicates in PAUP. The majority-rule consensus tree exhibits the same topology as the shortest tree found by exhaustive search (BP reported on Fig. 6). The neighbor-joining analysis (NJ and NJBOOT in MUST) provided the same topology and similar bootstrap proportions (Fig. 7). This second analysis confirms the first one: the sclerosponges *Astrosclera* and *Acanthochaetetes* belong to two different clades, a hadromerid clade and an axinellid clade. The hadromerid clade (*Cliona, Spirastrella, Acanthochaetetes*) is supported by 100% BP in both distance and parsimony analysis, and the internal topology is also supported by 92% BP and 88% BP for (*Spirastrella, Acanthochaetetes*) in distance and parsimony analysis. The axinellid clade (*Axinella, Astrosclera1, Astrosclera2*) is supported by 100% BP. The monophyly of the two *Astrosclera* individuals is supported by 100% BP; the individuals came from the same area of New Caledonia and do not represent the two populations, differing by the presence or absence of spicules, that occur respectively in the Indian Ocean and the Central Pacific (Vacelet, 1981; Ayling, 1982).

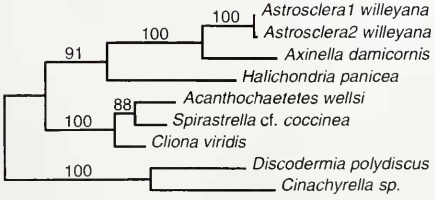

Figure 6. Phylogram obtained with PAUP by exhaustive analysis on full-length aligned sequences (914 bp) for 9 species using ACCTRAN optimization option. Tree length = 640, CI = 0.839, RI = 0.783, and G1 = −1.01. Bootstrap proportions (1000 replicates using Branch and Bound) are shown above internal branches.

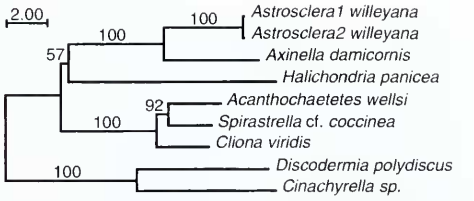

Figure 7. Phylogram obtained with MUST by neighbor-joining analysis on full-length aligned sequences (914 bp) for 9 species. Bootstrap proportions (1000 replicates by NJ analysis) are shown above internal branches.

Discussion

The Ischyrospongiae (Termier and Termier, 1973) hypothesis is falsified by the first analysis. The "coralline" sponge *Petrobiona massiliana* clearly belongs to the class Calcarea, whereas the two other calcified sponges, *Astrosclera* and *Acanthochaetetes,* are undoubtedly part of the Demospongiae. The class Ischyrospongiae is thus polyphyletic, as concluded previously from morphology, and should be abandoned.

Both current analyses demonstrate the polyphyly of the class Sclerospongiae, and it too should be abandoned in classification schemes. Furthermore, the two sclerosponges belong to different monophyletic clades, an axinellid one (*Axinella, Agelas, Astrosclera*) and a hadromerid one (*Cliona, Spirastrella, Acanthochaetetes*). Both clades are strongly supported in the two analyses. They are in complete agreement with the affinities indicated by spicule morphology and by cytology (Hartman and Goreau, 1970, 1975; Vacelet, 1981; Vacelet and Garrone,

1985; Reitner and Engeser, 1987; Boury-Esnault *et al.*, 1990). These results support the interpretation that the capacity to secrete a massive skeleton of calcium carbonate has developed several times during the course of the evolution of the Porifera (Vacelet, 1979, 1983, 1985; Wood *et al.*, 1989). Accordingly, these "coralline" sponges have to be classified in the Demospongiae: *Acanthochaetetes wellsi* in the order Hadromerida; and *Astrosclera willeyana* in the order Agelasida, which is considered by most recent authors as distinct from the order Axinellida, although closely related to it. The creation of a special order—the Tabulospongida—based on the presence of a calcareous skeleton (Hartman and Goreau, 1975) in *Acanthochaetetes wellsi* and its fossil relatives has no strong justification according to the present results.

At a lower taxonomic level, the classification of these sponges as belonging either within existing families to which they are closely related or in distinct families is still subjective. Pending analyses of other related sponges, the decision depends upon individual judgments about the size of the morphological gap needed to separate taxa and about the importance of the calcareous skeleton as a taxonomic character. In the case of *Acanthochaetetes*, we propose to classify the genus in the family Spirastrellidae Ridley and Dendy, 1886, in view of the spicular and cytological resemblances (periflagellar sleeve, central cell) and the low genetic distance between *Acanthochaetetes* and *Spirastrella* that is indicated by the present work. This hypothesis, which was already proposed by Reitner (1991), avoids the use of the family Acanthochaetetidae, which would be monogeneric at least in the Recent fauna. (We reject, however, on the grounds of morphology, Reitner's merging of the genus *Acanthochaetetes* with *Spirastrella*.) In the case of *Astrosclera*, we prefer to maintain the two families Astroscleridae Lister, 1900 (with five genera in the Recent, if merged with Ceratoporellidae), and Agelasidae Verril, 1907 (with one large genus). The genetic distance between *Astrosclera* and *Agelas*, as estimated by our sequences, is admittedly as low as for *Acanthochaetetes* and *Spirastrella*. However, the Agelasidae and the Astroscleridae differ by an important reproductive character: *Agelas* is oviparous (Liaci and Sciscioli, 1975; Reiswig, 1976), whereas *Astrosclera* is viviparous (Lister, 1900). Furthermore, the structure of the spongin fibers of *Agelas* (De Vos *et al.*, 1991), which are unique among the Demospongiae, is an important difference between *Agelas* and *Astrosclera*.

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